Genotyping of Polyomavirus BK by Real Time PCR for VP1 gene

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RESEARCH

Genotyping of Polyomavirus BK by Real Time PCR for VP1 Gene

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Abstract Polyomavirus BK latently persist in different sites, including the renourinary tract, and may reactivate causing nephropathy in renal transplant recipients or hemorrhagic cystitis in bone marrow recipients. Based on the sequence of the VP1 gene, four genotypes have been described, corresponding to the four serologically differentiated subtypes I–IV, with different prevalence and geographic distribution. In this study, the development and clinical validation of four different Real-Time PCR assays for the detection and discrimination of BKV genotypes as a substitute of DNA sequencing are described. 379 BK VP1 sequences, belonging to the main four genotypes, were aligned and "hot spots" of mutation specific for all the strains or isolates were identified. Specific primers and probes for the detection and discrimination of each genotype by four Real-Time PCR assays were designed and technically validated. Subsequently, the four Real-Time PCR assays were used to test 20 BK-positive urine specimens from renal transplant patients, and evidenced a prevalence of BK genotype I, as previously reported in Europe. Results were confirmed by sequencing. The availability of a rapid and simple genotyping method could be useful for the evaluation of BK genotypes prevalence and studies on the impact of the infecting genotype on viral biological behavior, pathogenic role, and immune evasion strategies.

Keywords Polyomavirus BK · Genotyping · Real-time PCR · Sequencing · Prevalence

Stefano Gambarino and Cristina Costa contributed equally to this work and share first authorship.

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Introduction

BK virus (BKV) belongs to the Polyomaviridae family and has a circular double-stranded DNA genome of about 5100 bp [1]. After primary infection that usually occurs in the childhood and, which is mainly asymptomatic, BKV remains latent at different sites, including the renourinary tract, B-cells, and brain [2]. Reactivation with viruria may occur, mainly in the context of immunosuppression, and potentially lead to nephropathy (BKV-associated nephropathy—BKVAN) in renal transplant recipients [3] or hemorrhagic cystitis in bone marrow transplant patients [4].

BKV is the only primate polyomavirus with serologically differentiated subtypes (I-IV) [5]. Jin and colleagues developed a genotyping method based on the amplification of the epitope region of the VP1 gene by polymerase chain reaction (PCR) and classified viral isolates in four different

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genotypes (BKV I-IV) corresponding to the serological ones [6].

The geographical distribution of BKV subtypes has been investigated in different studies [7–10], evidencing a higher prevalence for BKV I worldwide and a minor but consistent occurrence of BKV IV, in particular in South-East Asia. On the other hand, the prevalence of BKV II and BKV III is very low with a higher presence of BKV III in the African continent. This genotyping method is based on the presence of single nucleotide polymorphisms (SNPs) conserved between the various isolates belonging to each of the four genotypes. Many different biomolecular procedures have been developed to detect and discriminate SNPs [11–14]; among these, the ARMS PCR [15] uses the discriminatory power of the terminal 3′ nucleotide to obtain a successful amplification only for the DNA strand with a correct complementariness.

In this article, we describe the design of four different Real-Time PCR assays which can detect and discriminate among the four main BKV genotypes (I–IV) as a valid substitute of DNA sequencing.

Materials and Methods

BKV Sequences and Phylogenetic Analyses

379 BKV VP1 sequences, belonging to the main four genotypes (I–IV), were obtained from three different articles [8–10] by extrapolation from the server Nucleotide (www.ncbi.nlm.nih.gov/nucleotide/). Nucleotide positions from 1663 to 1912 (Dunlop strain complete genome, GeneBank Access no. V01108) were considered to obtain 250-bp length DNA sequences. Subsequently, the VP1 DNA sequences were aligned using the software Clustal X (i.e., the graphical version, with windows interface, of the bioinformatic software Clustal W). This software, that incorporates a novel position-specific scoring scheme and a weighting scheme for down weighting over-represented sequence groups, is used for multiple sequence alignment and phylogenetic analysis, with the possibility to draw phylogenetic trees. The software BioEdit was used to manipulate the alignment for a clearer sight of differences between the aligned sequences. BioEdit software is a user-friendly tool for post-alignment modifications, with the possibility to emphasize few sequences, cut and paste them, and make other evaluations, such as restriction mapping. The BKV VP1 sequences were then used for Phylogenetic analyses. Clustal X, that was utilized to make a neighbor-joining phylogenetic tree, and the free software NJplot were employed to display the resulting tree. The confidence of branching patterns of the neighbor-joining tree was determined by bootstrap analysis, using a set of 1,000 replicates.

Primers and Probes Design

The alignment was screened to find "hot spots" of mutation specific for all the strains or isolates belonging to each genotype (Fig. 1). In the absence of a specific single nucleotide mutation, the occurrence of a different nucleotide combination between the genotypes was considered. Every mutation was evaluated as a possible target for designing of a set of primers able to recognize all the BKV strains belonging to a specific genotype and to discriminate between the others by a mismatch at the 3′ primer ending. The parameters that were taken into consideration for selecting the "hot spots" and discriminating the primers design were the presence of a unique sequence shared between the strains belonging to one genotype but not to the others, the 60°C melting temperature, and the possibility to design a Real-Time PCR probe in the most conserved nucleotide region inside the hypothetic amplicon.

Primers and probes were designed with the help of the software Primer Express® v3.0 (Applied Biosystem, Cheshire, UK). The primers obtained were then analyzed with the Autodimer Software, an open-source tool that evaluates the hairpin and primer–dimer formation, with the aim of excluding the presence of factors that could alter the amplification efficiency. In order to include all the strains

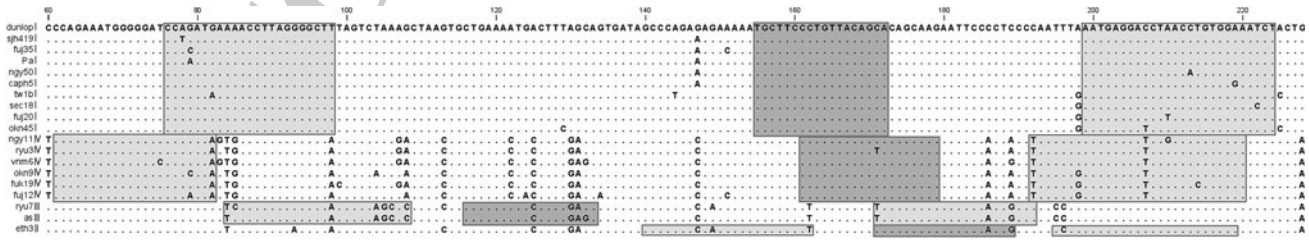

Fig. 1 Regions considered for the primer and probe design. Identical residues are represented as *dots*. *Light squares* indicate the primer regions. *Dark squares* represent the probe regions

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Table 1 Primers and probes for the four Real-Time PCR assays for BK genotyping

	Forward primers	Reverse primers	Probes
BK I	5′-CCAGATGAAAACCTTAGGGCTT-3′	5′-AGATTTCCACAGGTTAGGTCCTCATT-3′	5′-FAM-TGCTTCCCTGTTACAGCA-MGB-3′
	5′-CCTGATGAAAACCTTAGGGCTT-3′	5′-AGATTTCCACATGTTAGGTCCTCATT-3′	
	5′-CCACATGAAAACCTTAGGGCTT-3′	5′-AGATTCCCACAGGTTAGGTCCTCATT-3′	
	5′-CCAAATGAAAACCTTAGGGCTT-3′	5′-AGATTTCCACAGGTTAGATCCTCATT-3′	
	5′-CCAGATAAAAACCTTAGGGCTT-3′	5′-AGGTTTCCACAGGTTAGGTCCTCATT-3′	
		5′-AGATTTCCACAGGTAAGGTCCTCATT-3′	
BK II	5′-GCCCAGACAAAAAAATGCTTCCT-3′	5′-TCCACAGGTTAGGTCCTCATTTAGA-3′	5′-FAM-CACAGCAAGAATTCCACTG-MGB-3′
BK III	5′-TMACCTTAGGGCTATAGTCAGCAC-3′	5′-GGGCAGTGGAATTCTTGCTGTA-3′	5′-FAM-CTGAAAATGCCTTTGASA-MGB-3′
BK IV	5′-CCAGAAATGGGGGATCCAGATA-3′	5′-TTCCACAGGTTAGATCCTCATTTAAATTA-3′	5′-FAM-CTGTTACAGYACAGCAAG-MGB-3′
	5′-CCAGAAATGGGGGACCCAGATA-3′	5′-TTCCACAGGTTAGATCCTCATTCAAATTA-3′	
	5′-CCAGAAATGGGGGATCCAAATA-3′	5′-TTCCACAGGTCAGATCCTCATTTAAATTA-3′	
	5′-CCAGAAATGGGGGATCCACATA-3′	5′-TTCCACGGGTTAGATCCTCATTCAAATTA-3′	
CLONING	5′-GACAATCACATGCCTGGATAATG-3′	5′-TGGTAATGGACTAAGTGTGCGTTATTTTC-3′	

belonging to one genotype, many pairs of primers were designed and produced. Four Taqman® probes MGB (minor groove binding) labeled at the 5′ end with FAM (6-carboxyfluorescein), each being able to recognize one of the four target regions amplified by the different pairs of primers, were also designed. The list and sequences of primers and probes are reported in Table 1.

Real-Time PCR Optimization

Four different mixes for the amplification of each genotype were prepared. Different concentrations of primers and probe were evaluated: in particular, 200/200, 200/100, 100/200, 200/50, and 50/200 nM for primer forward/reverse, and 250 or 100 nM for probe. The reaction mixture contained 1× Master Mix (Platinum qPCR supermix—UDG with ROX [Invitrogen, Carlsbad, CA]). For this evaluation, isolates from BKV I (Dunlop strain) and BKV IV (kindly provided by Dr. Andi Krumbholz and Dr. Ellen Krautkramer), and ultramers that simulated BKV II and III sequences (comprehending a portion of isolates ETH3 and NEA27, respectively [Tema Ricerca, Bologna, Italy]), were used. Fifteen microliters of amplification mix were added to 5 µl of BKV DNA, obtaining a final volume of 20 µl. The procedure was performed and optimized on the 7300 Real-Time PCR System (Applied Biosystems). The following thermal profile was used: one cycle of decontamination at 50°C for 2 min, one cycle of denaturation at 95°C for 10 min, and followed by 45 cycles of amplification at: 95°C for 15 s, and 60°C for 60 s. The following parameters were considered for the evaluation: quick observation of the amplification, and good discrimination between the specific and aspecific amplification.

Plasmids and Standards

The BKV I Dunlop standard plasmid was kindly provided by Prof. Tiziana Musso. The BKV II and BKV III standard plasmids were produced starting from the ultramers (Tema Ricerca), then amplified with cloning primers (Table 1). The BKV IV standard plasmid was prepared starting from the BKV IV isolate J/2296/04 (kindly provided by Dr. Andi Krumbholz). The primers were used for producing a PCR product, then cloned using the pTOPO–TA cloning system (Invitrogen) and propagated in competent *Escherichia coli* TOP10 cells. After overnight culture, selected transformed clones were amplified by culture in liquid LB medium (10 g BACTOTRYPTONE, 5 g yeast extract, 171 mM NaCl, and 15 g/l agar, pH 7.5) containing 50 µg/ml of ampicillin. Plasmid DNA was extracted and purified using the Wizard Plus SV Miniprep DNA Purification System (Promega, Madison, WI). The plasmid concentration was estimated on spectrophotometric reading at OD260.

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Real-Time PCR Evaluation (Linearity, Efficiency, Interference Limit, Limit of Detection, Precision)

The four genotyping assays were evaluated using serial 10-fold dilutions of the target plasmid (ranging from 10^7 to 10 copies per reaction). The linearity was assessed by the correlation coefficient (R^2) of the standard curve obtained by plotting 10^7–10 standard copies per reaction with the four different tests. The efficiency was evaluated by the slope of the standard curve, using the formula $E = 10^{(-1/\text{slope})}$-1.

In order to discriminate between specific and aspecific amplifications, three repetitions of 10^7, 10^5, and 10^3, and 10 copies per reaction of the four standards were amplified with the four different genotyping mixes. The average threshold cycle (Ct) values of specific and aspecific amplifications were obtained. The background Ct was evaluated by the formula $Ct_{\text{lim}} = Ct_a - t_{0.01} \bullet \sigma_a/N$, where N is the number of observations, and $t_{0.01}$ is the tabulated value of Student's t for the 1% probability level and $N-1$ degree of freedom [16]. For the evaluation of the lowest genotype concentration that could be distinguished from the aspecific amplification of the other three genotypes, the formula $IL = 100/(2^{(Ct_s - Ct_{\text{lim}})} + 1$ was used, where Ct_s represents the specific Ct [17].

The limit of detection, defined as the lowest target quantity detectable, was also estimated by serial 10-fold standard dilutions. The precision of each test was calculated by the intra- and inter-test coefficients of variation, according to the formula $CV = 100 \bullet (\sigma/MC)$, where MC is the average measured concentration, and σ the correspondent standard deviation. Three repetitions for each standard dilution were considered in the same run or in different experiments, for the intra- and the inter-assay coefficients of variation, respectively.

Clinical Specimens

Twenty urine specimens resulting positive to a reference amplification assay (BKV Q-PCR Alert Kit [Nanogen, Buttigliera Alta, Turin, Italy]) were tested with the four amplification assays to assess the genotyping procedure. Automated DNA extraction was performed with the NucliSens EasyMAG platform (bioMeriéux, Marcy l'Etoile, France) from 1 ml of urine, according to the manufacturer's instructions, and eluted to a final volume of 50 µl. Five microliters of extracted specimen were then added to 15 µl of each genotyping mix. The results were analyzed using the System SDS software.

Sequencing Procedure

To confirm the results obtained by the Real-Time amplification, sequencing procedure was performed. The 20 specimens were amplified with primers BKV-1 (5′-GAA GTT CTA GAA GTT AAA ACT GGG-3′) and BKV-2 (5′-GTG GAA ATT ACT GCC TTG AAT AGG-3′) [9], obtaining an amplicon of 354 nucleotides within the VP1 gene (from 1663 to 2016, Dunlop reference numbering). The amplification mix contained 6 µl of GoTaq® HotStart Polymerase buffer 5× (Promega), 200 µM of each dNTP, 6 mM of MgCl2, 1 unit of GoTaq® HotStart Polymerase (Promega), and 20 pmol of BKV-1 and BKV-2 primers, respectively. The resulting amplicons were run on agarose gel (2% w/v) by electrophoresis, the gel was observed on an UV transilluminator UV and the bands cut. Subsequently, the bands were purified using the Nucleospin® Extract II (Macherey–Nagel, Düren, Germany). The purified PCR products were sequenced using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystem). In brief, a mix containing 8 µl of Terminator Ready reaction mix, 2 µl of PCR product, 3.2 pmol of inner primer (BKV-1 or BKV-2), and deionized water to obtain 20 µl final volume was prepared. Then, the Cycle sequencing was carried out on 9800 Fast Thermal Cycler (Applied Biosystem) with an initial denaturation step at 96°C for 1 min, followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min.

The Cycle sequencing product was then purified by Ethanol/EDTA/Sodium Acetate precipitation and loaded onto the ABI PRISM 31 Genetic Analyzer (Applied Biosystem). Forward and reverse sequences were aligned with the ClustalX sotware and then compared to BKV sequences.

Results

The best amplification was obtained with the following concentrations of primers forward/reverse: 100/200, 200/200, 200/200 and 200/100 nM for genotypes I, II, III, and IV, respectively, and with a probe concentration of 250 nM; however, the amplification efficiencies were 104, 104, 97, and 88%, respectively. The R^2 coefficient was 0.996, 0.999, 0.995, and 0.999 for BK I, II, III, and IV genotype amplifications, respectively.

Subsequently, the Real-Time PCRs were tested for the discrimination of the four genotypes. Three repetitions of 10^7, 10^5, and 10^3, and 10 copies/reaction of the target sequence of the main four BKV genotypes were employed to compare the amplification and distinguish between specific and aspecific amplifications using the four mixes (Table 2). As regards the BKV I genotype mix, this resulting product was able to amplify the genotype I-specific target, and only BKV II and IV genotype mixes cross-recognize the specific genotype I target at 10^7 copies/reaction (both), and at 10^5 copies/reaction (only the BKV II genotype mix). However, a

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Table 2 Aspecific and specific means *C*t for each genotyping mix

	Specific mean *C*t	Aspecific mean *C*t		
	BK I	BK II	BK III	BK IV
10^7	17.36 (0.07)	32.16 (0.34) [32.06]		
10^5	24.12 (0.07)	38.8 (0.16) [38.75]		38.13
10^3	30.9 (0.51)			
10	36.12			
	BK II	BK I	BK III	BK IV
10^7	16.85 (0.04)		36.57 (0.62) [36.39]	32.2 (0.36) [32.10]
10^5	23.21 (0.04)			35.94 (0.65) [35.75]
10^3	29.97 (0.19)	36.14	37.74	36.51 (0.94) [36.24]
10	36.03		40.89	37.15
	BK III	BK I	BK II	BK IV
10^7	17.78 (0.16)			35.62 (0.9) [35.36]
10^5	24.2 (0.16)			
10^3	30.84 (0.03)			
10	37.62 (0.63)		36.91	38.61 (0.74) [38.40]
	BK IV	BK I	BK II	BK III
10^7	17.67 (0.03)		36.12 (0.81) [35.89]	35.88 (0.59) [35.71]
10^5	24.74 (0.01)			
10^3	32.3 (0.19)	36.78		
10				

The values in the round brackets are the standard deviations of the repetitions for each dilution; in the square brackets the Ct_{lim} are reported (see text for explanation)

gap of approximately 14.8–14.68 Ct_s separated the specific and aspecific amplifications; moreover, no aspecific amplification was evidenced for lower dilution.

BKV II genotype mix that resulted was able to amplify the specific BKV II target sequence;' however, this was also amplified by the other three BKV discriminating mixes. Only the amplification of the BKV II-specific target with the corresponding mix was linear, and the threshold crossing appeared at least 15 cycles earlier for BKV II mix at 10^7 copies/reaction compared to the other amplification mixes, decreasing to six cycles at 10^3 copies/reaction. The amplification with BKV II mix at lower BKV II genotype-specific target concentration was considered not discriminating, because of the overlapping Ct with BKV III and IV amplification mixes. The BKV III target was recognized by the corresponding mix with a linear amplification; however, 10^7 copies/reaction were recognized also by the BKV I mix, although a mean gap of 17.84 Ct_s was observed between specific and aspecific amplifications. An undistinguishable specific amplification was seen with a concentration of BKV III of 10 copies/reaction, because of the simultaneous amplification with BKV II, III, and IV mixes with a similar *C*t. The BKV IV target showed a specific recognition using the corresponding mix, although the target was also amplified with BKV II and III mixes at 10^7 copies/reaction, with a mean gap of 18.21 and 18.45 Ct_s between specific and aspecific amplifications for BKV II and III mixes, respectively. However, at a concentration of 10^3 copies/reaction, the target was also recognized by BKV I mix with a mean gap of 4.48 mean Ct_s.

Using the same data to evaluate the specific and aspecific amplifications, a Ct_{lim} was calculated for each genotyping amplification, as an interference limit (Table 3). The precision was calculated with the intra- and inter-test coefficients of variation (Table 4).

In order to establish the limit of detection for the four different genotyping amplifications, 10-fold dilutions of the targets (ranging from 10^7 to 10 copies/reaction) were amplified with the above four methods. The limit of detection differed between the four genotyping amplification procedures and was as follows: 10^2 copies/reaction for BKV IV and 10 copies/reaction for BKV I, II, and III, each. However, only BKV III procedure showed a sensitivity of 10 copies/reaction, while for the others assay, it was of 100 copies/reaction.

Results for the clinical specimens are reported in Table 5. In brief, the amplification procedures yielded positive results

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Table 3 Interference limit

	10^7 (%)	10^5 (%)	10^3 (%)	10 (%)
I–II	0.00375	0.00393		
II–III	0.00013			
II–IV	0.00257	0.01674	1.27874	
III–IV	0.00051			36.83754
IV–>II	0.00033			
IV–III	0.00037			

The lowest limit of quantity of BKV-specific genotype, expressed in percentage, distinguishable from the background (aspecific amplification). In the case of no reported percentage, sporadic or no background amplification was observed

Table 4 Intra- and inter-assay coefficients of variation for dilutions of 10^7, 10^5, and 10^3, and 10 copies/reaction

	10^7 (%)	10^5 (%)	10^3 (%)	10 (%)
Coefficient of intra-assay variation				
I	0.40	0.29	1.65	
II	0.23	0.17	0.63	
III	0.89	0.66	0.10	1.67
IV	0.16	0.04	0.59	
Coefficient of inter-assay variation				
I	0.92	2.70	0.84	1.85
II	1.19	0.15	1.45	0.99
III	1.30	1.51	0.91	2.92
IV	3.34	2.20	2.26	

in 16 cases; in particular: 12716 BKV I, 1/16 BKV II, 1716 BKV III, and 1/16 BKV IV. The DNA sequencing confirmed the results obtained with the Real-Time PCR assays for all the samples. One specimen (#4464) showed a concomitant amplification for BKV I and BKV IV, with overlapping Ct_s; sequencing identified it as BKV I genotype.

In most of the cases, the viral load found with the developed methods differed from that obtained with the reference method, with a percentage of variation ranging from −288.4 to 89.9% (Table 5). Furthermore, more aspecific amplifications were observed in clinical specimens. In fact, the sample #2998 showed two aspecific plots (BKV I and BKV II), while no BKV I amplification was observed with a 10^7 copies/reaction plasmid dilution. Nevertheless, the occurrence of aspecific amplifications did not impact on the genotyping performance of the Real-Time PCRs, as the gap between the curves of amplifications remained wide.

Discussion

In this study, a Real-Time PCR-based method for genotyping of polyomavirus BK was developed. By hemagglutination inhibition tests, four main BKV serotypes were described [5], based on the differences in a short amino acidic sequence on VP1 protein, probably being responsible for the serotypical differences among BKV variants [18]. Correspondingly, four main BKV VP1 genotypes were found, and their nucleotide sequences analyzed [18]. On the basis of this conserved region, 379 VP1 sequences were collected from three different articles [8–10] investigating the prevalence of BKV subtypes in different countries, including Germany, Japan, and China. These sequences were aligned to determine which nucleotide residues were conserved within each genotype with the aim to design a Real-time PCR assay based on the principle of the Amplification Refractory Mutation Assay (ARMS).

A complete list of single nucleotide polymorphisms in the total BKV genome for each genotype has been reported [19]. The 379 aligned VP1 sequences presented concordant residues with those previously listed by Luo and colleagues [19], confirming the uniqueness and conservation of these polymorphisms among the genotypes.

It has been reported [19] that genotyping on large T antigen (LTA) is preferable, in particular, because LTA is a larger region with more informative sites compared to the 327 bp of VP1 region considered by Jin and colleagues [6]. However, in this study, we chose to use the VP1 region instead of LTA as we aimed to produce powerful discriminating Real-Time PCRs taking into consideration the data published in the literature. Indeed, a higher number of sequences is banked in public domain servers for VP1 compared to LTA, thus permitting a more detailed primers-and-probes design for the detection of a higher number of sequences and corresponding polymorphic residues. In this study, four Real-Time PCR assays, each one that can amplify specifically a specific genotype, were designed, and four different amplification mixes were produced to avoid a too high concentration of primers in a single well, that could determine dimer formation or an increase in the aspecific performance. In fact, in the presence of high viral loads, aspecific amplifications might appear, and these were detectable as a low quantity amplification with other genotype mixes; anyway, the aspecific amplification was easily recognizable by the wide gap between the specific and aspecific plots. Moreover, the introduction of Ct_{lim} and interference limit gave further clues about discrimination. Clearer results were available for 10^7–10^5 copies/reaction dilutions, while 10^3 caused the lowest dilution with an acceptable genotyping performance. For this reason, a result comparable to 10^3 was set as genotyping limit for all the four Real-Time PCRs, and all those results with a lower quantity were not accepted.

As regards the clinical validation of the four genotyping assays, the distribution of BKV genotypes was evaluated in

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Table 5 BK genotyping in clinical specimens

N°	ID	Reference copies/ml	BKV I (*Ct*)	BKV II (*Ct*)	BKV III (*Ct*)	BKV IV (*Ct*)	Genotype	Copies/ml
1	2998	$>5 \times 10^6$	31.63	33.82		11.59	IV	$>5 \times 10^7$
2	3068	4237203	19.57	36.36		32.78	I	$>5 \times 10^7$
3	3204	569						
4	3206	$>5 \times 10^6$	12.01	26.65		25.34	I	$>5 \times 10^7$
5	3207	2413	30.60				I	5379 (−122.9%)
6	3607	126470	29.38	36.56			I	12800 (89.9%)
7	3619	1542						
8	3965	502131	24.72	43.06		38.04	I	351153 (30.1%)
9	4011	868						
10	4063	4158	30.14				I	7458 (−79.4%)
11	4326	495	34.78				I	<500
12	4398	49606	29.73	43.09		38.53	I	9981 (79.9%)
13	4405	2472943	34.93	19.80		32.37	II	1220410 (50.6%)
14	4464	8689	**36.59**			**36.69**	I	<500
15	4674	6441	29.22	42.79		42.64	I	14342 (−122.7%)
16	4712	2393	30.58			37.65	I	5456 (−128%)
17	4714	<59	36.17				I	<500
18	4723	$>5 \times 10^6$	12.19	28.37		26.11	I	$>5 \times 10^7$
19	4758	<59						
20	4768	44562		31.50	29.14		III	173076 (−288.4%)

Viral loads (expressed in copies/ml) of positives samples for the reference method, positivity to each amplification procedure (expressed in *Ct*), genotype and quantities (expressed in copies/ml) are reported. *Bold* numbers shows Ct_s of overlapping curves

urine specimens; this type of sample was chosen for the higher mean viral load in comparison to serum samples [2]. However, clinical samples with a low reference viral load were not always detected. Most of the specimens were infected by BKV I (80%), while BKV II, III, and IV were infrequent. The prevalence of the four genotypes was concordant with the literature [20]. BKV IV, that is more prevalent in East Asia [8], is found also in Europe [9] in a percentage comparable to the one observed by us; however, notwithstanding the uncommon prevalence, both BKV II and BKV III were detected in two different samples; the results were confirmed by the subsequent VP1 DNA sequencing.

Considering viral load of different genotypes, a high divergence was observed between the genotyping assays and the reference method, with a variability ranging from −288.4 to 89.9%. These data highlighted the problem exposed by Hoffman and coll. [21]: in the presence of various genotypes, difference in probes and primers for the BKV quantification led to a substantial disagreement between assays. This was confirmed in our study, between the reference and the genotyping amplifications.

In conclusion, the four Real-Time PCRs that were produced were able to detect and discriminate the main four BKV genotypes. Compared to classical DNA sequencing, these procedures are more rapid and simpler, since they are single PCR assays with no purification steps; results are available in approximately 2 h. One drawback could arise because of the need of at least 10^3 copies/reaction for the genotyping; in these cases, a PCR assay employing external primers, or other protocols, would be necessary to increase the target sequence amplifiable, although this should also increase the time required for genotyping.

The comparison between genotyping procedures on clinical samples and plasmid dilutions evidenced some differences, including the detection of aspecific plots during the testing of clinical samples, and the lack of similarity with the quantities reported by the reference amplification procedure. This could be caused by the primers-and-probes nature, amplifying Large T (reference method) and VP1, and by the presence of polymorphism that alter the efficiency of amplification, as previously suggested [21]. Nevertheless, the four Real-Time PCRs developed in this study should not be considered quantitative methods, as the procedures were not tested for the quantification of a broad number of isolates. The availability of a rapid and simple genotyping test could be useful for the evaluation of BKV genotypes' prevalence. and studies on the impact of the infecting genotype on viral biological behavior, pathogenic role, and immune evasion strategies.

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